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In situ localization of the transcripts of a homeobox gene in the honeybee *Apis mellifera* L. (Hymenoptera)

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Summary. We have isolated and characterized a homeobox-containing gene from the honeybee *Apis mellifera*. Its homeobox region shows a high degree of sequence similarity to the homeobox of the *Drosophila* gene *Deformed* (*Dfd*). At the DNA level 82% of the basepairs are the same, whereas the putative amino acid sequences are identical between the bee and the fruitfly genes. Similarity is also present 5′ and 3′ to the homeobox. Using this isolate as a probe we have performed in situ hybridization on sections from blastoderm-stage embryos of the honeybee *Apis mellifera*. In early blastoderm stages we found a rather irregular pattern of labelled nuclei. In middle stages we found silver grains over each nucleus and also over the cytoplasm in a belt of blastoderm cells in the prospective gnathal region. These results indicate that the *Deformed* genes from honeybee and fruitfly are homologous both with respect to their DNA sequence and their spatial and temporal pattern of expression during embryogenesis.

Key words: *Apis mellifera* – Homeobox genes – *Dfd* – In situ hybridization – Blastoderm

Introduction

A common feature of many metazoa is the subdivision of their body into homologous segments. In *Drosophila melanogaster* it has been shown that two classes of genes are required for establishing the characteristic segmentation pattern. Segmentation genes establish the basic metameric units and homeotic genes determine the identity of each unit (Nüsslein-Volhard et al. 1982). Many of these genes share a similar 180-bp nucleotide sequence, the homeobox (McGinnis et al. 1984a; Scott and Weiner 1984). Sequence similarity of homeobox proteins with yeast mating-type sequences and with several bacterial DNA-binding proteins (Sheperd et al. 1984; Laughon and Scott 1984) has led to the proposal that the homeo domain may bind to DNA. DNA binding studies (Desplan et al. 1985) have further supported this hypothesis. The general significance of the homeobox was shown by the discovery of these sequences in vertebrates; thus far homeobox sequences have been isolated from frog, rat, mouse and man (Carrasco et al. 1984; Müller et al. 1984; Falzon et al. 1987; McGinnis et al. 1984c; Colberg-Poley et al. 1985; Levine et al. 1984).

Using Southern blot analysis and molecular cloning we have recently shown that homeobox sequences are also present in the honeybee *Apis mellifera* (unpublished results). Due to its large size (1.7 mm) and slower development, the honeybee egg offers some advantages to monitor early patterns of gene expression more precisely. Development until hatching takes about 70 h at 35° C (Nelson 1915; Schnetter 1935; Du Praw 1967; Fleig and Sander 1986). The blastoderm stage begins about 8 h after egg deposition and lasts until 33 h, when gastrulation starts; between 10 h and 34 h no mitoses can be observed in the scanning electron microscope (SEM) (Fleig and Sander 1985). Thus the absolute, as well as the relative, duration of the blastoderm period is long when compared to that of *Drosophila melanogaster* (Turner and Mahowald 1976; Campos-Ortega and Hartenstein 1985) and many other insects.

One of the homeoboxes we have found shows strong sequence similarity to the homeobox of the *Deformed* (*Dfd*) gene of *Drosophila melanogaster*. In the fruitfly, *Dfd* is involved in establishing head structures (Morgan et al. 1925; Regulski et al. 1987). In situ hybridizations at blastoderm and germ-band stages of *Drosophila* reveal a belt of label in an area which approximately contains the anlagen of the maxillary and the mandibular segments (McGinnis et al. 1984a; Harding et al. 1985; Gehring 1987; Chadwick and McGinnis 1987; Martinez-Arias et al. 1987). Our in situ hybridizations at mid-blastoderm stages of the honeybee show a similar belt of cytoplasmic transcript accumulation. This indicates homology of the *Drosophila* and the *Apis* genes, not only at the DNA level, but also with respect to time and region of gene activity during embryogenesis.

Material and methods

Materials. Honeybees (*Apis mellifera*) and honeybee eggs were collected from bee colonies kept at the University of Freiburg, Germany. Radionucleotides were purchased from Amersham and all enzymes from Biofinex or Boehringer.

General methods. Preparation of genomic DNA was as described earlier (Walldorf et al. 1984). Restriction endonuclease digestions, gel electrophoresis of DNA, labelling of DNA fragments, Southern transfer experiments and the construction of a genomic library were performed as described by Maniatis et al. (1982).

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Library screening. A library of *Sau*3A partially digested genomic *Apis mellifera* DNA cloned into the *Bam*HI site of the EMBL4 phage lambda vector (Frischauf et al. 1983) has been used for screening. 1.5×10^5 phages (5 genome equivalents) were screened under low stringency conditions at 37° C. The hybridization buffer consisted of 43% deionized formamide, 5 × SSC (1 × SSC is 0.15 *M* NaCl, 15 m*M* sodium citrate), 4 × Denhardt's solution, 0.1% SDS, 0.1% sodiumpyrophosphate, 20 µg/ml tRNA and 50 µg/ml heparin. The filters were washed twice for 30 min each at 50° C in 2 × SSC.

DNA sequencing. All DNA sequencing procedures were carried out using the M13 cloning (Messing and Vieira 1982) and chain termination sequencing (Sanger et al. 1977) methods. Both strands of the DNA were sequenced.

In situ hybridization. Eggs were collected every 2 h from a honeybee queen (*Apis mellifera carnica*) encaged on a single empty honeycomb. They were incubated in darkness at 35° C and high humidity up to the appropriate age and developmental stage (Fleig and Sander 1986). After dechorionation with sodium hypochlorite the eggs were fixed for 1 h with glutaraldehyde in the heptane phase of a glutaraldehyde/*n*-heptane mixture (Zalokar and Erk 1977). Cryosectioning and in situ hybridization of the sections was performed as described for *Drosophila* by Hafen et al. (1983). As hybridization probe we used the 0.34-kb *Sac*I-*Sal*I fragment (Fig. 2). It includes the greater part of the homeobox and the M repeat 3′ of the box (Fig. 3). Labelling of the probe with tritium was done by nick translation. Time of exposure was 5 weeks in a dry chamber at 4° C.

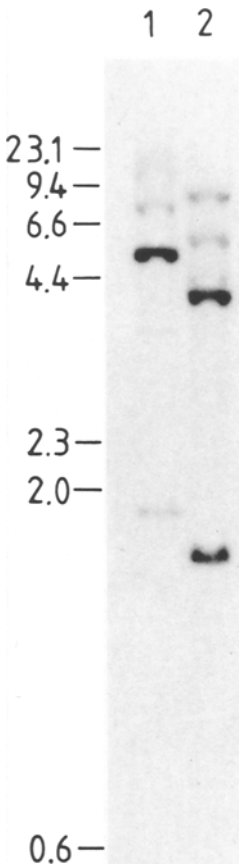


Fig. 1. Homeobox similarity in the honeybee genomic DNA. Genomic DNAs were digested with *Eco*RI and hybridized under low stringency conditions with a *Drosophila Dfd* homeobox probe (*Dfd* 250-bp *Hpa*II fragment). Lane 1, 2.5 µg *Drosophila* DNA; lane 2, 7.5 µg *Apis mellifera* DNA. Size markers are lambda *Hin*dIII fragments

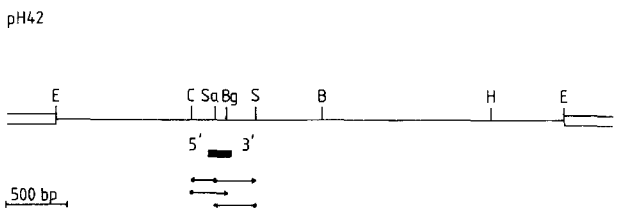


Fig. 2. Restriction map of clone pH42. The *black box* identifies the region containing homeobox similarity, 5′ and 3′ is indicated. Restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; C, *Cla;* E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sa, *Sac*I. *Arrows* below the map represent sequence determinations

Results

Using Southern blot analysis we have previously shown that at least four *Eco*RI fragments of honeybee DNA hybridize with the *Drosophila* homeobox probes *Antp, ftz, Scr* and *Dfd* (Walldorf et al. unpublished results). An example of such a genomic Southern blot is shown in Fig. 1. Here the homeobox sequence of the *Drosophila* gene *Dfd* was used as a probe. In the lane with genomic *Drosophila* DNA several bands are visible, the strongest being the *Deformed* fragment itself, whereas the others represent cross-hybridizing fragments of other homeobox containing genes. Using genomic honeybee DNA the strongest band corresponds to a size of 4.2 kb, a 1.5-kb band being also quite strong and bands of 5.5 kb and 9.0 kb are less intense. Since the strongest band always has the highest sequence similarity to the probe, we considered the 4.2-kb fragment to be closely related to the *Drosophila Dfd* homeobox, whereas the other bands represent other homeobox-containing genes which are more diverged (Walldorf et al. unpublished results). For further analysis we used the lambda phage B4, which was isolated during a screen of a genomic honeybee library under low stringency conditions with the *Antp* probe (Walldorf et al. unpublished results). This clone contains the 4.2-kb *Eco*RI fragment mentioned above, which hybridizes with both *Antp* and with *Dfd*. The fragment was subcloned into pUC8 and a restriction map established (Fig. 2). We found the homeobox sequence similarity to be located within a 500-bp *Cla-Sal*I fragment whose DNA sequence was determined.

The fragment has a complete homeobox sequence which is 82% identical to a cDNA sequence of the *Drosophila Dfd* gene's homeobox (Fig. 3). More striking is the fact that the homeoboxes of the *Dfd* gene and of the honeybee gene (termed *H42*) code for an identical amino acid sequence; in addition, sequence similarity extends nine amino acids beyond both the 5′ and 3′ ends of the box (Fig. 3). This translates into a total of 78 identical amino acids in *Dfd* and *H42*. 5′ of the homeobox the sequence similarity

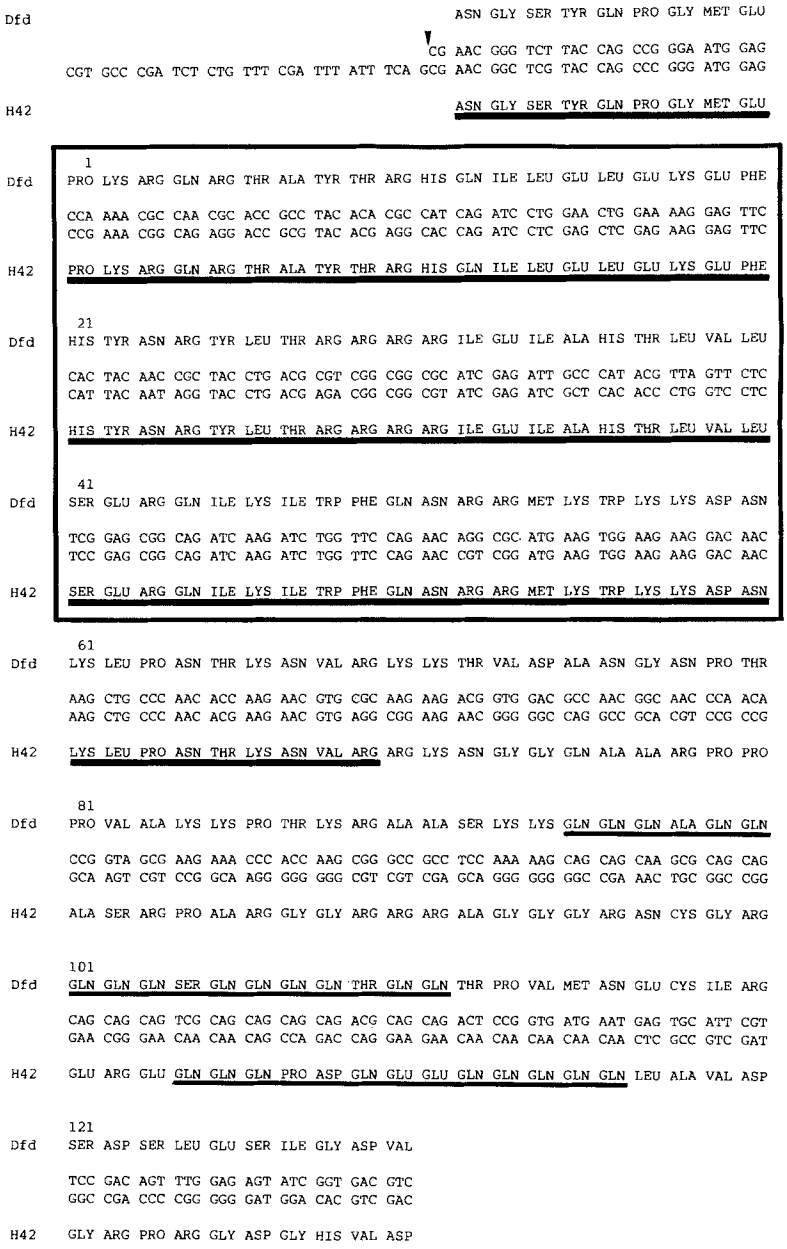
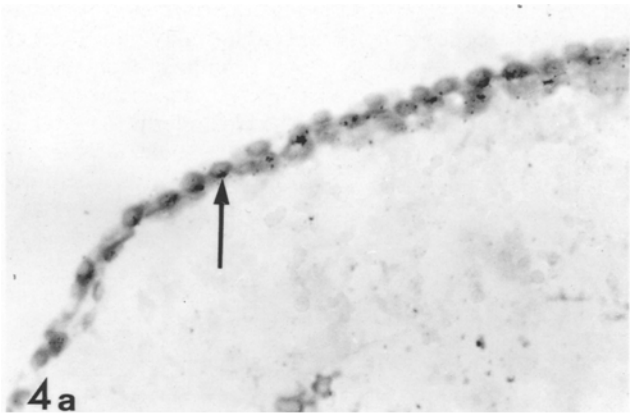


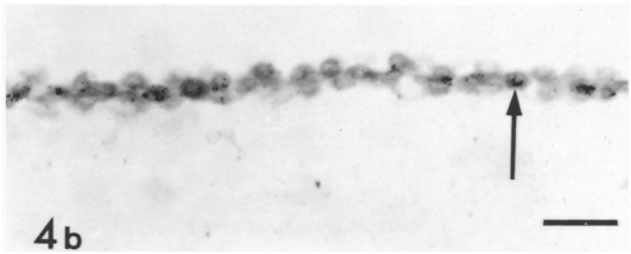
Fig. 3. DNA and protein sequence comparison of *Drosophila Dfd* and *Apis H42*. DNA and protein sequences of *H42* are aligned with the comparable region from *Dfd* (clone cDf 41, Regulski et al. 1987). Putative splice sites are indicated by *arrow*. The homeobox region is *boxed* and contiguous stretches of identical amino acids are underlined (*thick bar*). M repeat regions are also underlined (*thin bars*)

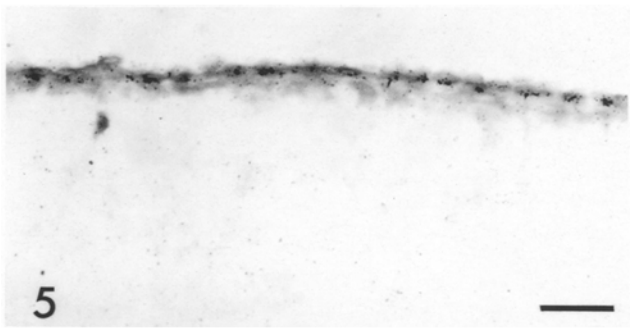
stops at the position where *Dfd* has an intron. Since we find a perfect consensus splice site at this position in *H42*, it is likely that it also has an intron 5′ to the homeobox. Nine amino acids 3′ to the homeobox the degree of sequence similarity drops although we still have an open reading frame in both clones. The only similarity in this region is due to M repeat sequences (McGinnis et al. 1984a; Wharton et al. 1985), located in both cases at a similar distance from the homeobox. Since the sequence comparisons favoured the hypothesis that the *H42* honeybee gene is a true *Dfd* homologue, we tested this by comparing the temporal and spatial distribution of *H42* transcripts in *Apis mellifera* with that of *Deformed* transcripts in *Drosophila melanogaster*.

In early blastoderm stage sections (17 h) clusters of silver grains are found only over the blastoderm nuclei (Fig. 4). In this stage the blastoderm temporarily seems to be bilayered, although the cells are still open towards the yolk sac (Nelson 1915; Fleig and Sander 1985). The volume of the bottle-shaped blastoderm cells is largely taken up by their nuclei. The label is not equal over the whole nucleus but is concentrated over a restricted region (Fig. 4). We find label also over the nuclei of vitellophages inside the yolk system. In the 18-h embryo the blastoderm nuclei form a monolayer again (Nelson 1915; Fleig and Sander 1985) (Fig. 5). In this stage, too, label is found only over restricted parts of the nuclei (Fig. 5), which are now located in the distal part towards the periphery of the blastoderm cells. Not all nuclei are labelled but we find a rather patchy pattern of labelled and unlabelled nuclei. During the next 4 h (no mitoses are observed in this stage) increasing numbers of nuclei show silver grain labelling.

In sections of mid-blastoderm stage (24–28 h) we find label over every nucleus in blastoderm cells and vitellophages (Fig. 6). As in younger stages, the labelled area is smaller than the nucleus itself. The nuclei of the extra-em-







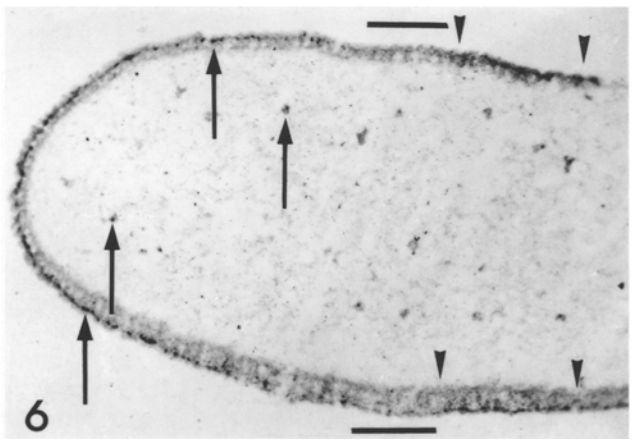
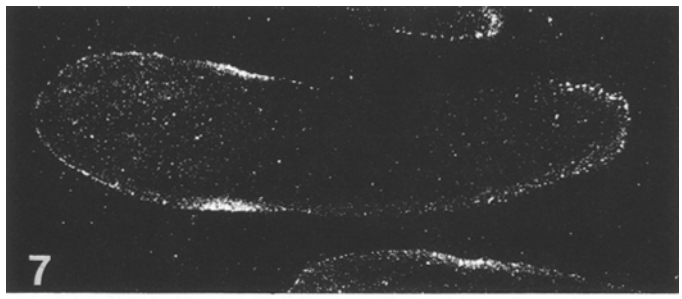


Fig. 4a, b. Details of a section from an early blastoderm stage. **a** Anterior, **b** near posterior end. Label can be seen over the blastoderm nuclei, which are arranged in two layers. Label is restricted to a defined part of the nuclei (*arrows*). Age 17 h after egg laying, bar 10 μm

Fig. 5. Section of an early blastoderm stage embryo. The nuclei have returned to a single-layered arrangement close to the periphery. Only nuclei are labelled, the blastodermal cytoplasm shows no label. Age 18 h after egg laying, bar 10 μm



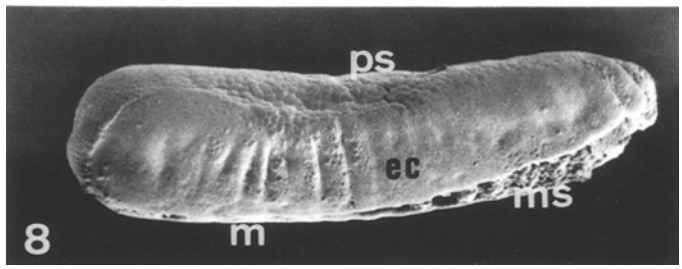


Fig. 7. Dark field photograph of a section from an intermediate blastoderm stage. The blastodermal cytoplasm is labelled between 60% and 75% egg length (posterior pole, to the *right*, is 0%). Label in posterior pole region is not cytoplasmatic. Age 26 h after egg laying, total length 1.4 mm

Fig. 8. Scanning electron micrograph of a gastrulation-stage embryo, lateral view, anterior to the *left*. Segmental grooves can be seen with differing clarity in head, thorax and abdomen. *m*, Maxillary segment; *ec*, ectoderm; *ms*, mesoderm; *ps*, preserosa. Age 35 h after egg laying, total length 1.2 mm

bryonic dorsal parts of the blastoderm (anlagen of dorsal strip, serosa and amnion) do not differ in labelling from those of the embryonic lateral and ventral parts which will give rise to ectoderm and mesoderm/endoderm, respectively. But now we find label also over the entire cytoplasm of all blastoderm cells in a belt between 60% and 75% egg length (posterior pole is 0%) (Fig. 7). Comparison with a gastrulating embryo (Fig. 8), which is already developing segmental grooves, shows that this region should contain the anlagen of the mandibular, maxillary and perhaps labial segments, or at least parts of them. The signal intensity over the cytoplasm and nuclei of the cells in the belt is much higher than over the nuclei outside the belt. Labelling is very strong in the middle of this belt and fades out towards its borders. The more strongly labelled central part of the belt (about 65%–70% egg length) is 15–20 cells wide, which equals about two segments (segment width is about 8–10 cells once grooves become visible). It may contain the complete maxillary and at least part of the mandibular segment anlage (compare Figs. 7, 8). The labelled belt runs all around the circumference of the blastoderm. It can be seen in both the embryonic and extra-embryonic regions and even in the mid-dorsal parts, where during the next hours the blastoderm will be interrupted temporarily (dorsal strip, Nelson 1915; Fleig and Sander 1985) (Fig. 7). La-

Fig. 6. Anterior part of section in Fig. 7. Every nucleus in both blastoderm and yolk is labelled (*arrows*). Anterior to the belt of cytoplasmic labelling (between *arrowheads*) an area of weaker labelling in the nuclei can be seen (bars)

bel over the nuclei located just outside the labelled belt seems to be weaker than in nuclei further away from the belt (Fig. 6).

In late blastoderm stage sections (age 30 h, about 3 h before the onset of gastrulation) we no longer observed label over the vitellophage nuclei. In this stage we find a pattern of narrow peripheral belts of labelling, but due to insufficient material we cannot as yet establish any spatial relationship between the labelled belts and individual segments or segmental grooves.

Discussion

Homeobox sequences of the *Antp* class show a high degree of conservation in different species, whereas flanking sequences are not conserved. Among vertebrates, sequence similarity outside the homeobox has been shown in a few cases (Boncinelli et al. 1985). The same is true for homeobox genes in different insects (Walldorf et al. unpublished results). Sequence similarity outside the homeoboxes between insects and vertebrates has up to now been described only for the *Drosophila* genes *engrailed* (*en*) (Joyner and Martin 1987) and *Deformed* (*Dfd*) (Regulski et al. 1987). Our sequence data demonstrate that the honeybee clone *H42* is a *Dfd* homologue. As expected, homology is higher between the two insect species than between insects and vertebrates. A region coding for 78 amino-acids including the homeobox is absolutely identical in *Dfd* and *H42*. This is the first case of a homeobox being identical in two different species (of two different orders: Dipterans and Hymenopterans). The identical locations of splice sites 5′ to the box and the presence of an M repeat 3′ to the homeobox are further hints for a high degree of conservation of the gene structure in both species. Since we only have genomic clones we cannot compare *Dfd* to the whole honeybee homologue, but still this high degree of conservation is striking.

The pair of genes from the two species express a similar spatial and temporal pattern of activity, at least during the blastoderm stage. The basic body organisation of both *Drosophila melanogaster* and *Apis mellifera* is similar. Both are long germ developers, which rapidly subdivide the embryonic (ventral) part of the blastoderm into the complete set of future segmental units (see Krause 1939; Sander 1976). Their individual segments are easily homologized although specific differences exist; for instance, head involution and reduction of the last two abdominal segments are specialities of *Drosophila melanogaster* and other cyclorrhapic Dipterans, whereas a specific trait of *Apis mellifera* consists of the absence of germ-band elongation and retraction.

The labelled belt of *H42* expression in the mid-blastoderm stages of the honeybee embryo is positioned as *Dfd* expression is in the fruitfly during cellular blastoderm (McGinnis et al. 1984a; Harding et al. 1985; Gehring 1987; Chadwick and McGinnis 1987; Martinez-Arias et al. 1987). The comparison of SEM preparations of early gastrula stages with the belt of labelling in the blastoderm stage section shows that in the honeybee the belt is equivalent to about two segment anlagen. This coincides with the *Dfd* results in *Drosophila melanogaster* (McGinnis et al. 1984a; Harding et al. 1985; Gehring 1987; Chadwick and McGinnis 1987; Martinez-Arias et al. 1987).

Nuclei in all regions of the blastoderm, as well as the vitellophages, carry label for at least 7 h before we find label over the cytoplasm in the blastoderm region between 60% and 75% egg length. These findings may indicate that the transcript is stored somehow for many hours in the nuclei, or even in a restricted part of them, before being released only in specific areas of the blastoderm; or a rapid degradation outside the nuclei prevents higher accumulation of the mRNA in the cytoplasm. In *Drosophila* no such nuclear storage of *Dfd* transcripts is reported. It might have been overlooked because of the short duration of the blastoderm stage, but in the case of the *Drosophila* gene *fushi tarazu* (*ftz*), clusters of silver grains are first detectable over the nuclei during blastoderm formation.

At first, the *H42* gene becomes active in all nuclei, as the nuclear labelling indicates, but subsequently its transcripts accumulate in a blastodermal belt. Within the belt the labelling is cytoplasmic as well as nuclear. The appearance of this belt could be due to other genes repressing the release of *H42* transcripts into the cytoplasm in certain regions and stages. Alternatively, the transcript may be degraded immediately outside the nuclei but in the belt region the rate of transcription may exceed that of degradation. The number of silver grains is much higher over the cells of the belt (cytoplasm and nuclei) than over those in the other regions of the embryo (nuclei only), and this would support the second assumption.

Since our probe contains the M repeat 3′ to the homeobox (Fig. 3), we cannot rule out some non-specific labelling due to cross-hybridization with other M-repeat sequences. In this case, however, we would expect a more or less irregular pattern or higher background. The regularity and intensity of the signals over the nuclei as well as the distinctness and high intensity of the cytoplasmic labelling in a region and developmental stage comparable with the *Dfd* gene's transcription in the *Drosophila* embryo argues against this possibility.

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